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Primary Prevention of Coronary Arterial Thrombosis with the Factor Xa Inhibitor rTAP in a Canine Electrolytic Injury Model

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Summary

The antithrombotic efficacies of the coagulation factor Xa inhibitor recombinant tick anticoagulant peptide (rTAP) and heparin were compared in a canine model of left circumflex (LCX) coronary artery electrolytic lesion. Intravenous infusions of saline (controls), rTAP (50 μg/kg/min continuous infusion) or heparin (200 U/kg bolus followed by 2 U/kg/min continuous infusion) were started 60 min prior to the initiation of LCX coronary artery electrolytic injury (150 μA continuous anodal current). All 6/6 saline-treated control animals developed occlusive thrombi at 49.8 ± 13.6 min after the initiation of vessel injury, and possessed a residual thrombus mass of 20.7 ± 3.3 mg. In the rTAP treatment group, 4/6 preparations developed occlusive thrombi, but with times to thrombosis delayed significantly compared to both the saline control as well as to the heparin treatment group (202.7 ± 28.9 min; $p < 0.01$ to both saline and heparin groups). The remaining 2 rTAP-treated preparations remained patent despite the continued electrical stimulation of the coronary vessel for 5 h. Residual thrombus mass in the rTAP treatment group was reduced markedly compared to the saline control group (4.4 ± 1.0 mg; $p < 0.01$). Heparin infusion resulted in a modest but statistically insignificant delay in occlusive LCX coronary artery thrombosis compared to saline controls, with all 6/6 heparin-treated preparations occluding at 79.7 ± 16.5 min after the initiation of vessel injury. Residual thrombus mass in heparin-treated animals, however, was reduced compared to saline controls (9.4 ± 1.4 mg; $p < 0.01$). These results support a pivotal role for fXa in the process of arterial thrombosis, as well as the feasibility of inhibiting fXa as an effective strategy for the primary prevention of arterial thrombosis.

Introduction

The serine protease factor Xa (fXa) catalyzes the formation of thrombin, which is the penultimate enzymatic activation step in the amplification phase of the blood coagulation process. The feasibility of the antagonism of fXa activity as an antithrombotic strategy has been evaluated in experimental models using active site-blocked fXa (1-3), the aprotonin mutant 4C2 (4), the non-peptide fXa inhibitor DX-9065a (5, 6), and, most extensively, the naturally-derived fXa inhibitor tick anticoagulant peptide, TAP (7-9). Previous studies with recombinant TAP (rTAP) have demonstrated significant efficacy vs thromboplastin-induced disseminated intravascular coagulation in Rhesus monkey (8), stasis-induced venous thrombosis in rabbits (10, 11) and thrombus deposition on in situ foreign surfaces, including Dacron grafts positioned in femoral arteriovenous shunts in baboons (12) and copper coils inserted within femoral arteries of dogs (13). rTAP also enhanced reperfusion and maintained post-thrombolysis vessel patency when administered adjunctively with tPA in a canine model of coronary artery electrolytic injury (14, 15). One aspect of the antithrombotic profile of rTAP which has not been addressed, however, is that of primary prevention of arterial thrombosis in an injured vessel. Therefore, the present investigation was conducted as a proof of principle study, assessing the effect of inhibition of fXa activity with intravenous rTAP, with comparison to an intravenous loading plus infusion regimen of heparin, on thrombus formation occurring in response to electrolytic injury of the intimal surface of the left circumflex coronary artery in dogs. The results of this study demonstrate significant potential for fXa inhibition in the primary prevention of occlusive coronary arterial thrombosis.

Materials and Methods

Surgical Preparation

The surgical preparation of the canine model of left circumflex (LCX) coronary artery electrolytic injury has been described previously (16). Male or female purpose-bred mongrel dogs (10-16 kg) were anesthetized with sodium pentobarbital (35 mg/kg i.v.), and were ventilated with room air using a positive pressure ventilator (Harvard Apparatus, S. Natick, MA). The right femoral artery and vein were cannulated for the measurement of mean arterial pressure (Statham P23ID, Gould Inc, Cleveland, OH) and for drug administration, respectively. The left external jugular vein and cephalic vein were cannulated for the continuous infusion of 5% dextrose in saline and for drug administration, respectively. The heart was exposed via a left thoracotomy at the 5th intercostal space. The left circumflex (LCX) coronary artery was isolated proximal to the first obtuse marginal branch, and dissected for a distance of approximately 2 cm. The vessel was instrumented, proximal to distal, as follows: electromagnetic flow probe (Model 501, Carolina Medical Electronics Inc, King, NC), stimulation electrode, adjustable mechanical occluder and a silk snare. The stimulation electrode was constructed from a 26-gauge stainless steel hypodermic needle tip attached to a 30-gauge Teflon-insulated silver-coated copper wire. The mechanical occluder was constructed of stainless steel with a stainless steel screw (2 mm diameter), which could be manipulated to control vessel circumference. Before the administration of test agents, the occluder was sufficiently tightened around the artery to just eliminate the reactive hyperemic response

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without affecting resting LCX coronary artery blood flow. Continuous records of systemic blood pressure and mean and phasic LCX coronary artery blood flow were displayed on a model 7E polygraph (Grass Instrument Co., Quincy, MA). Zero flow and hyperemic flows were determined by occluding the LCX coronary artery distal to the flow probe for 20 s with the snare. LCX coronary artery electrolytic injury was induced by the application 150 μA continuous anodal direct current to the stimulation electrode. Direct electrical stimulation was delivered using a Grass constant current unit (model CCU1A) coupled to a Grass stimulus isolation unit (model SIU5) and powered by a Grass stimulator (model S48; driving voltage 100 V) connected to the intraluminal LCX coronary artery stimulation electrode.

Experimental Protocol

Thirty minutes after surgical preparation, three groups of 6 dogs each were randomized to treatment with intravenous saline, rTAP (50 μg/kg/min continuous infusion) or heparin (200 U/kg bolus followed by 2 U/kg/min continuous infusion). Sixty minutes after the start of treatment, the process of electrolytic injury of the LCX coronary artery was initiated by the application electrical current to the intimal surface of the proximal LCX coronary artery. Treatment infusions and electrical stimulation of the coronary artery were maintained until LCX blood flow decreased to and remained at zero for a 30 min period in those preparations which developed occlusive thrombi, or until 5 h of continuous coronary artery electrical stimulation had elapsed in those preparations which remained patent. Similarly, intracoronary thrombi were retrieved and wet thrombus mass obtained after a 30 min period of zero LCX blood flow or after five h of coronary artery electrical stimulation. Buccal mucosal template bleeding times were determined, and blood samples for the determination of plasma rTAP concentration, activated partial thromboplastin time (aPTT), platelet count and the assessment of ex vivo platelet aggregation responses to ADP and collagen were obtained at multiple time points during the protocol using the methods described below.

Buccal Mucosal Template Bleeding Times

Buccal mucosal template bleeding times were measured using a modification of previously described methods (17) with a SIMPLATE bleeding time device (Organon Teknika Corporation, Durham, NC). Uniform incisions were made on the mucous membrane of the inner upper lip of the dog, and the duration of bleeding was timed to a maximum of 15 min.

Activated Partial Thromboplastin Time

Effects upon the intrinsic clotting pathway were assessed by determination of the activated partial thromboplastin time (aPTT). Two ml of arterial blood were drawn into a syringe containing 0.2 ml of 3.8% trisodium citrate solution. The blood was centrifuged for 10 min at 2000 × g. The plasma was removed and stored on ice for later assay. aPTTs were determined using an automated clot timer (Electra 800®, Medical Laboratory Automation, Mt. Vernon, NY) and commercially available reagents (American Dade, Aquada, Puerto Rico).

Ex Vivo Platelet Aggregation

Arterial blood was drawn into a plastic syringe containing one part 3.8% trisodium citrate to nine parts blood. Platelet-rich plasma (PRP) was obtained by centrifugation of this mixture at 150 × g for 10 min at room temperature. Platelet count was adjusted to 3×10^8/ml. All aggregation studies were performed in an aggregometer (Chrono-Log Corp., Havertown, PA) using 0.25 ml of PRP in a siliconized cuvette stirred at 1000 rpm. Platelet-rich plasma (PRP) was prewarmed for 3 min at 37° C before addition of agonist. ADP (10 μM in the presence of 1 μM epinephrine) and collagen (10 μg/ml in the presence of 1 μM epinephrine) were added to the cuvette and the change in light transmittance was measured to the point where the tracing reached a plateau or after a period of 3 min. Platelet aggregation was expressed as % light transmittance with platelet poor plasma representing 100% light transmittance.

Determination of Plasma rTAP Concentrations

Plasma concentrations of rTAP were determined by measuring the fXa inhibitory activity in plasma samples as described previously in a chromogenic assay using purified human fXa and the substrate Spectrozyme Xa (American Diagnostica) (8).

Materials

rTAP was prepared as described previously (18) and determined to be >98% homogeneous by a number of analytical criteria including reverse phase high performance liquid chromatography, mass spectral analysis and amino acid terminal sequence analysis. Bovine heparin sodium was obtained from the Upjohn Co, Kalamazoo, MI.

Statistical Analysis

Data are expressed as the mean ± S.E.M. Within group comparisons were performed using a repeated measures analysis of variance followed by a Dunnett's test for multiple comparisons. Among group comparisons were performed using a one-way analysis of variance followed by a Dunnett's test for multiple comparisons.

Results

Left Circumflex (LCX) Coronary Artery Thrombosis

All 6/6 saline-treated control animals developed occlusive thrombi at 49.8 ± 13.6 min after the initiation of vessel injury, and possessed a residual thrombus mass of 20.7 ± 3.3 mg. In the rTAP treatment group, 4/6 preparations developed occlusive thrombi during the study, with 2 rTAP-treated preparations remaining patent despite the continued electrical stimulation of the LCX coronary artery for a period of 5 h. Times to occlusive thrombus formation in the 4 rTAP-treated preparations which occluded (i. e., excluding those rTAP preparations which remained patent for the duration of the protocol) were delayed markedly compared to both the saline control as well as to the heparin treatment group (202.7 ± 28.9 min; $p < 0.01$ to both saline and heparin groups). Residual thrombus mass in the rTAP treatment group was reduced markedly compared to the saline control group (4.4 ± 1.0 mg;

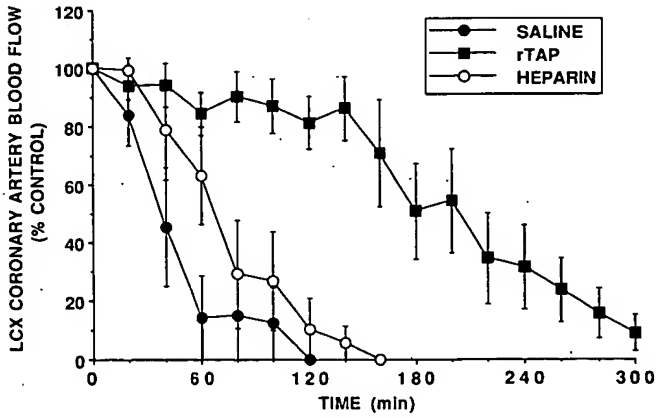

Fig. 1 Left circumflex (LCX) coronary artery blood flow (% of control, pre-vessel injury value) in the saline control, rTAP (50 μg/kg/min continuous i.v. infusion) and heparin (200 U/kg i.v. bolus followed by 2 U/kg/min continuous i.v. infusion) treatment groups. Time 0 represents initiation of electrical stimulation (150 μA anodal) of the LCX coronary artery. Treatments were started 60 min prior to the initiation of LCX coronary artery electrical stimulation. Data are mean ± S.E.M. with n = 6. Two of six rTAP-treated preparations remained patent for the entire 300 min period of LCX electrolytic injury

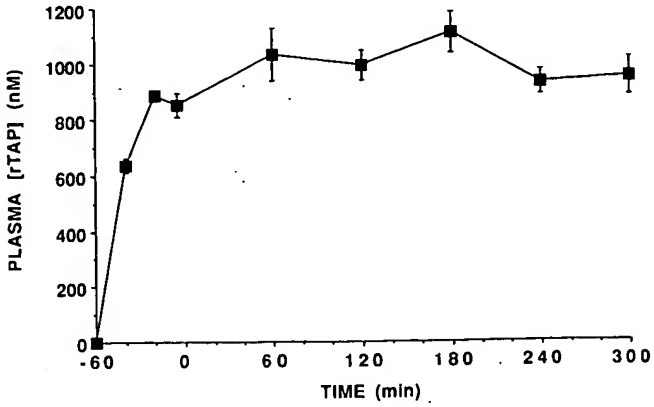

Fig. 2 Plasma rTAP concentrations in animals administered 50 μg/kg/min continuous i.v. rTAP. Time 0 represents initiation of electrical stimulation (150 μA anodal) of the LCX coronary artery. rTAP infusion was started 60 min prior to the initiation of LCX coronary artery electrical stimulation. Data are mean ± S.E.M. with n = 6

p <0.01). Heparin infusion resulted in a modest but statistically insignificant delay in occlusive LCX coronary artery thrombosis compared to saline controls, with all 6/6 heparin-treated preparations occluding at 79.7 ± 16.5 min after the initiation of vessel injury. Residual thrombus mass in heparin-treated animals, however, was reduced compared to saline controls (9.4 ± 1.4 mg; p <0.01). Fig. 1 depicts LCX coronary artery blood flows in all three treatment groups during the course of the study, and demonstrates the superior efficacy of rTAP compared to heparin in maintaining coronary blood flow during continuous intimal injury.

Plasma rTAP Concentrations

Fig. 2 depicts plasma rTAP concentration as a function of time in the six animals administered 50 μg/kg/min i.v. rTAP, beginning 60 min prior to the initiation of coronary artery electrical stimulation and injury. A plasma rTAP concentration of 852 ± 43 nM was achieved 5 min prior to the initiation of coronary artery injury, and plasma rTAP concentrations ranging from 927 ± 45 to 1106 ± 74 nM were maintained during continuous electrical stimulation of the coronary vessel. No obvious differences in rTAP concentrations were noted between those rTAP-treated preparations which developed occlusive coronary artery thrombi and those which remained patent for the duration of the study. Plasma clearance of rTAP, determined from the plateau phase of the plasma rTAP concentration-time relationship, ranged from 5.1 to 8.3 (mean 7.3 ± 1.2) ml/min/kg.

Hemostatic and Hemodynamic Parameters

Table 1 summarizes the effects of intravenous saline, heparin and rTAP infusions on sinus heart rate, mean arterial pressure, buccal mucosal template bleeding times and activated partial thromboplastin times (aPTT) at baseline and at 55 min, 120 min and 240 min after the initiation of treatments (i.e., pretreatment, 5 min before, 60 and 180 min after the initiation of LCX coronary artery injury, respectively). The latter time point (240 min after initiation of treatment) constitutes the final point in the protocol for which data were available for all 6 preparations entered into the rTAP treatment group. Sinus heart rate was unchanged in the heparin and rTAP treatment groups, and was reduced modestly in the saline control group. Mean arterial pressure

Table 1 Effect of intravenous saline (0.11 ml/min), heparin (200 U/kg + 2 U/kg/min) and rTAP (50 mg/kg/min) infusions of sinus heart rate, mean arterial pressure, buccal mucosal template bleeding time and activated partial thromboplastin time (aPTT) in the anesthetized canine model of left circumflex coronary artery electrolytic lesion

Parameter/Sample Time	Saline	Heparin	rTAP
Sinus Heart Rate (bpm)			
Baseline	183.0±6.0	154.0±2.7	159.0±7.7
55 min post treatment start	177.5±5.3	152.0±2.6	157.8±8.8
120 min post treatment start	151.8±4.8* (n=4)	155.6±3.1 (n=5)	158.8±8.9
240 min post treatment start	---	---	160.3±8.2
Mean Arterial Pressure (mm Hg)			
Baseline	100.3±10.0	89.0±5.8	103.3±9.0
55 min post treatment start	102.7±10.0	90.5±8.5	96.3±5.8
120 min post treatment start	73.4±19.7 (n=4)	93.0±5.8 (n=5)	79.9±5.7*
240 min post treatment start	---	---	81.8±4.0
Template Bleeding Time (min)			
Baseline	1.8±0.1	1.6±0.1	1.8±0.1
55 min post treatment start	1.9±0.2	2.5±0.1*	3.2±0.3*
120 min post treatment start	1.9±0.1 (n=4)	2.5±0.1* (n=5)	3.7±0.3*
240 min post treatment start	---	---	4.0±0.4*
aPTT (sec)			
Baseline	9.5±0.2	9.8±0.1	9.6±0.2
55 min post treatment start	9.9±0.2	86.3±16.9*	11.4±0.4
120 min post treatment start	10.6±0.3* (n=4)	85.3±20.7* (n=5)	12.2±0.6*
240 min post treatment start	---	---	12.6±0.7*

Data are mean ± S.E.M. with n = 6 unless otherwise indicated. Reductions in n and missing values at later time points in the saline and heparin treatment groups reflect preparations which developed occlusive coronary artery thrombosis and were removed from study for determination of thrombus mass.
*p <0.05 compared to baseline value by analysis of variance, followed by Dunnett's test for multiple comparisons.

was reduced modestly and comparably in the saline control and rTAP treatment groups over the course of the protocol, and was unchanged in the heparin treatment group. Buccal mucosal template bleeding times were unaltered in the saline control group (maximal 1.1 ± 0.1-fold increase above baseline), and were elevated moderately in the heparin and rTAP treatment groups (maximal 1.6 ± 0.1- and 2.3 ± 0.1-fold increases above baseline, respectively). It is noteworthy that 1.6-fold elevations in template bleeding time were observed at 55 and 120 min after the initiation of heparin infusion, while comparable 1.7- and 2.0-fold increases in template bleeding time were observed at 55 and 120 min after the initiation of rTAP infusion. Elevations in template bleeding time modestly exceeding 2.0-fold were observed when rTAP infusions were ≥180 min in duration, at which time most of the vehicle- and heparin-treated animals had been removed from the protocol due to the development of occlusive coronary artery thrombosis. aPTTs were elevated only slightly in the saline control and rTAP treatment groups (maximal 1.1 ± 0.1- and 1.3 ± 0.1-fold increases above baseline, respectively), whereas aPTT increased markedly (range 8.6 ± 2.0- to 11.5 ± 2.7-fold increases above baseline) in the heparin treatment group during the course of the study. Platelet counts were unaltered in all three treatment groups, and there were no important effects (i.e., no inhibition in extent of aggregation ≥20% compared to pretreatment response) on ex vivo platelet aggregation responses to ADP and collagen.

Discussion

The canine model of coronary artery electrolytic lesion has been widely utilized in the acute assessment of novel antithrombotic interventions, both for prevention of primary thrombosis (16, 18-29) as well

as for facilitation of thrombolytic therapy (14-16, 29-38). Previous light and scanning electron microscopic assessments of electrolytic injury in canine coronary artery, consistent with internal characterization of this preparation, have revealed that electrical stimulation of the intimal surface of the coronary vessel results in extensive disruption of the endothelial lining, with thrombus present in the area of vessel wall in contact with the stimulating electrode consisting primarily of platelet aggregates, with some erythrocytes and fibrin (36, 39, 40). The present investigation using this acute coronary artery thrombosis model constitutes a proof of principle study demonstrating protection against primary arterial thrombosis via inhibition of fXa activity, with the i. v. infusion of the fXa inhibitor rTAP significantly delaying or completely preventing occlusive arterial thrombosis despite continued electrical injury of the vessel. rTAP retarded arterial thrombus formation in the absence of marked changes in heart rate, mean arterial pressure and hemostatic function.

Varying degrees of thrombogenic insult, resulting from differences in the intensity and duration of electric current used to injure the coronary vessel, have been utilized in previous investigations employing the canine model of coronary artery electrolytic lesion. Conditions identical to those used in the present studies (150 μA continuous stimulation for 5 h) were used in one previous investigation from this laboratory (29). Other studies have utilized a shorter duration of stimulation (e.g., 150 μA for 3 h) (16), a lower intensity of stimulation (e.g., 100 μA for 5 h) (26, 27), or both lower intensity and shorter duration of stimulation (e. g., 100 μA for 1 h) (28). Given the relatively severe conditions of injury used in the present study, the ability of rTAP to markedly delay and, in two preparations, completely prevent thrombotic occlusion despite continuous high amperage stimulation of the vessel for 5 h is considered indicative of significant antithrombotic activity, thereby demonstrating the utility of fXa inhibition as an antithrombotic strategy.

For comparative purposes, the 50 μg/kg/min i.v. infusion regimen used in the present study represents an intermediate dose level relative to previous in vivo assessments of rTAP. Lower dose rTAP infusions have been used to prevent increases in plasma fibrinopeptide A levels occurring in response to thromboplastin-induced activation of systemic coagulation in Rhesus monkeys (20 μg/kg/min) (8), to inhibit thrombus deposition onto Dacron grafts positioned into baboon arteriovenous shunts (12.5-25 μg/kg/min) (12) and within copper coils inserted into canine femoral arteries (2.5-8 μg/kg/min) (13), and to prevent thrombotic reocclusion following tPA-mediated thrombolysis of occluded coronary vessels in the canine electrolytic injury model (10-20 μg/kg/min) (15). Conversely, shorter term, higher dose rTAP infusions have been used adjunctively to accelerate and increase the incidence of tPA-mediated reperfusion of occluded LCX coronary arteries in the canine electrolytic injury model (100-200 μg/kg/min) (14, 15). The dose of rTAP required to demonstrate antithrombotic efficacy in varying experimental preparations therefore is model-dependent, and most likely reflects the nature and severity of the thrombogenic insult in each preparation.

In contrast to the significant efficacy displayed by rTAP in the present studies, an i.v. loading plus continuous infusion regimen of heparin sufficient to markedly elevate aPTT to "supratherapeutic" levels (8.6-11.5-fold above baseline) was ineffective in delaying or preventing occlusive coronary arterial thrombosis although residual thrombus mass was reduced, presumably due to a limitation of clot extension. The lack of efficacy of heparin in previous experimental thrombosis studies in varying models (31-33, 41-43) as well as the equivocal efficacy of heparin in some clinical settings (44-47) has been attributed to the inability of the heparin-antithrombin III complex to access and inhibit clot-bound thrombin as well as to the neutralization of heparin activity by products of platelet activation such as platelet factor 4 (48, 49). Likewise, the heparin-antithrombin III complex is unable to directly access fXa assembled in the prothrombinase complex (50), consistent with the lack of antithrombotic activity manifest by heparin vs the fXa inhibitor rTAP in the present study. One previous study employing the canine coronary artery electrolytic lesion model has reported an i. v. heparin loading plus infusion regimen (80 U/kg + 0.5 U/kg/min) effective in delaying occlusive thrombosis (28). The apparent discrepancy in effectiveness for heparin between the latter and present study is likely due to the lesser severity of thrombogenic insult employed in the latter study (100 μA for 1 h) (28) compared to the present study (150 μA for 5 h). Other antiplatelet and anticoagulant interventions also have displayed antithrombotic efficacy in the canine coronary artery electrolytic injury model, most notably small peptide (16) and nonpeptide (29) inhibitors of platelet glycoprotein (GP) IIb/IIIa, monoclonal antibodies directed against the platelet GP IIb/IIIa (26), and direct, antithrombin III-independent inhibitors of thrombin (27, 28). As noted above however, key elements of the experimental protocols such as the duration and/or intensity of electrical current applied to the coronary artery, as well as the measurement of hemostatic parameters such as template bleeding times have varied among the laboratories conducting these studies. Therefore, it is not possible to directly compare the results of these studies and discern clear efficacy vs safety advantages among these mechanistically diverse interventions.

In summary, the fXa inhibitor rTAP significantly delayed or prevented the development of occlusive arterial thrombi in the present canine model of coronary artery electrolytic injury. rTAP inhibited coronary arterial thrombosis in the absence of marked changes in hemodynamic and hemostatic function. In contrast, heparin was ineffective in inhibiting coronary arterial thrombosis in this setting. These results support a pivotal role for fXa and the amplified generation of thrombin by the prothrombinase complex in the process of arterial thrombosis, as well as the feasibility of inhibiting fXa as an effective strategy for the primary prevention of arterial thrombosis.

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